A sensitive and reliable stripping voltammetric method was developed to determine chlorotetracycline drug. This method is based on the adsorptive accumulation of the complex of this drug with Zn (II) at a hanging mercury drop electrode and then a negative sweep was initiated, which yield a well defined cathodic peak at –928 mV versus Ag/AgCl reference electrode. To achieve high sensitivity, various experimental and instrumental variables were investigated such as supporting electrolyte, pH, accumulation time and potential, scan rate, frequency, pulse amplitude, convection rate and working electrode area. The monitored adsorptive current was directly proportional to the concentration of chlorotetracycline and it shows a linear response in the range from 1 x 10^{-7} to 1.4 x 10^{-6} mol l^{-1} of chlorotetracycline (correlation coefficient = 0.999) and the detection limit (S/N=3) is 7.2 x 10^{-9} mol l^{-1} at an accumulation time of 40 sec. The developed ADSV procedure shows a good reproducibility, the relative standard deviation RSD% (n=8) at a concentration level of 5 x 10^{-7} mol l^{-1} of chlorotetracyline and 1 x 10^{-6} mol l^{-1} of Zn^{2+} was 3.4%, whereas the method accuracy was indicated via the mean recovery of 99%±1.0. Possible interferences by several substances usually present in the pharmaceutical formulations have been also evaluated. The applicability of this approach was illustrated by the determination of the chlorotetracycline in pharmaceutical preparation and biological fluids such as serum and urine.

Keywords: Adsorptive stripping voltammetry, HMDE, chlorotetracycline, urine, serum.
Introduction

Of the most commonly used instrumental techniques, electroanalytical approach is the one of choice, and stripping voltammetric method has provoked particular interest because it is currently the most sensitive and widely used electrochemical technique. Its possibility of applications cover many fields ranging from environment, pharmaceutical and clinical to food and industrial samples. Many of the adsorptive stripping voltammetric (AdSV) approach features such as sensitivity, selectivity, simplicity and versatility attributed to the combination of an effective preconcentration step based on non-electrolytic adsorptive accumulation process with an advanced measurement procedures such as DP or SW (1-5). Unlike conventional stripping approaches (anodic and cathodic stripping voltammetry), which are based on an electrolytic nature of preconcentration step, AdSV approach in contrast is based on adsorptive accumulation of the analyte on the electrode at open circuit with no charge transferred. Consequently, for a wide range of surface-active organic and inorganic species, which cannot be preconcentrated electrolytically, the adsorption approach allows these analytes to be interfacially accumulated on the electrode and hence analysed. There have been many reviews devoted to emphasize and illustrate the wide spectrum and scope of AdSV applications and potentialities in the analysis of metal ions (6,7) organic analytes (8) and pharmaceutical drugs and biomedical compounds (9,10). Chlorotetracycline is a tetracycline antibiotic, and was the first tetracycline to be discovered. In veterinary medicine, it is commonly used to treat conjunctivitis in cats and it is used to overcome bacterial kinds which happen for humans. Chlorotetracycline is soluble in distilled water, its molecular weight is 515.35 g/mol and molecular structure C22H23N2O8Cl.HCl (11-12). The chemical structure of this drug and the mechanism for the electrochemical reduction process of its complex with Zn (II) ions are shown in Figure 1. owing to the biomedical importance of chlorotetracycline it has been analysed in pharmaceutical formulations and biological samples by various analytical methods such as Flow Injection Methods (13-16), spectrophotometry (17), fluorimetry (18) and liquid chromatography HPLC (19). In addition, chlorotetracycline and other tetracycline have been determined by adsorptive stripping voltammetry with linear sweep (20). However, to the best of our knowledge, the square wave voltammetric behavior of chlorotetracycline and thus its square-wave stripping voltammetry (SW-AdSV) have not been performed and reported so far. Consequently, the aim of this work was to develop more sensitive, reliable and simple AdSV procedure for the determination of chlorotetracycline in complex biological media and pharmaceutical formulations.

![Figure 1. The chemical structure of Chlorotetracycline and the mechanism of the electrochemical reduction process for its complex with Zn (II).](image_url)

Experimental

Apparatus:

All adsorptive stripping measurements were carried out with 797 VA computrace (Metrohm, Switzerland) in connection with Dell computer and controlled by VA computrace 2.0 control software. Stripping voltammograms were obtained via a hp deskjet 5150 printer. A conventional three electrode system was used in the hanging mercury drop electrode (HMDE) mode. Chromatographic determinations of the investigated drug were obtained by HPLC instrumental model LC-20AT Shimadzu (made in Japan) in connection with Dell computer. The obtained chromatograms were printed via a hp LaserJet 1020 printer. pH values were measured with Metrohm 632 pH meter. Biohit adjustable micropipette (AU), and Brand adjustable micropipette, were used to measure microliter volumes of the standard solutions.

Reagents:

All chemicals used were of analytical reagent grade and were used without further purification.
Chlorotetracycline (made in UK, Winlab) stock solution of 1×10^{-2} mol l^{-1} were prepared by dissolving the appropriate amount of chlorotetracycline hydrochloride in distilled water in 10 ml volumetric flask. Also, zinc stock solution of 1×10^{-3} mol l^{-1} was prepared by dissolving the appropriate amount of zinc nitrate in distilled water in 25 ml volumetric flask. Those stock solutions were stored in the dark and under refrigeration in order to minimize decomposition. Standard solutions of this drug with lower concentrations were prepared daily by diluting the stock solutions with distilled water. Britton-Robinson supporting buffer (pH \approx 2, 0.04M in each constituent) was prepared by dissolving 2.47 g of boric acid (winlab, UK) in 500 ml distilled water containing 2.3 ml of glacial acetic acid (BDH, UK) and then adding 2.7 ml of ortho-Phosphoric acid (Riedal-deHaen, Germany) and diluting to 1 L with distilled water. In addition, phosphate supporting buffer [0.1 M NaH_{2}PO_{4}(winlab, UK) and 0.1 M H_{3}PO_{4}] was prepared by dissolving 12.9 g of NaH_{2}PO_{4} and 6.78 g of H_{3}PO_{4} in 1000 ml distilled water. Acetate supporting buffer (0.02 M in each constituent) was prepared by dissolving 1.68 g of sodium acetate (winlab, UK) in 500 ml distilled water containing 1.12 ml of acetic acid and diluting to 1 L with distilled water. Finally, carbonate supporting buffer (0.1 M in each constituent) was prepared by dissolving 10.6 g of sodium carbonate (made in UK, BDH) and 8.4 g of sodium hydrogen carbonate (winlab, UK) in 1L distilled water. While, ammonia buffer was prepared by dissolving 4.5 g of ammonium chloride in 20 ml of distilled water and then adding 35 ml of concentration ammonia and diluting to 1 L with distilled water.

**Procedure:**

The general procedure adopted for obtaining adsorptive stripping voltammograms was as follows: A 10 ml aliquot of B-R supporting buffer (unless otherwise stated) at the desired pH (e.g. 2.5) was pipetted into a clean and dry voltammetric cell and the required standard solutions of chlorotetracycline and metal ion were added. The test solutions were purged with nitrogen for 5 minutes initially, while the solution was stirred. The accumulation potential of −0.4 V vs. Ag/AgCl was applied to a new mercury drop while the solution was stirred for 40 seconds. Following the preconcentration period, the stripping was stopped and after 20 seconds had elapsed, cathodic scans were carried out over the range 0.0 to −1.1 V. All measurements were made at room temperature.

**Preliminary Observations:**

When the differential pulse polarographic behavior was investigated for chlorotetracycline/Zn (II) complex in Britton-Robinson buffer at pH 2.5, a broad polarographic wave at E_{p} = -0.950 V was observed and this obtained polarographic wave is probably due to the electrochemical reduction of zinc ion (Zn^{2+}) [see figure 2], to zinc metal (Zn^{0}). A proposed mechanism for the electrochemical reduction of this electroactive metal is given in Figure 2. This mechanism suggesting that the electrochemical reaction is an irreversible process, an assumption which was confirmed by cyclic voltammetric measurement at a scan rate of 50 mV s^{-1} of chlorotetracycline in B-R buffer (pH 2.5). As can be seen from Figure 2, an anodic peak was observed on the measured cyclic voltammogram, resulting probably from oxidation amino group in chlorotetracycline drug to nitro group.

![Cyclic voltammogram of 5 x10^{-5} mol l^{-1} chlorotetracycline and 5 x10^{-2} mol l^{-1} Zn^{2+} in pH 2.5 B-R buffer, scan rate 50 mV s^{-1}.](image)

In order to obtain a voltammetric peak with better definition and higher sensitivity, a HMDE was used to study the adsorptive prosperities of chlorotetracycline/Zn (II) complex. The AdSV behavior of the complex was investigated in various supporting electrolytes at different pH values. This Drug/Zn complex yielded a well-developed and defined AdSV peak corresponding to the electroactive zinc ion at peak potential of −0.93 V. A
typical adsorptive stripping voltammogram for 5x 10^-7 mol l^-1 chlorotetracycline and 1x 10^-6 mol l^-1 zinc ion in B-R buffer is shown in Figure 3, which illustrates a well observed electrochemical peak indicating a strong and readily adsorption process at the surface of the working electrode.

Parameters Affecting the Adsorptive Stripping Response:
Effect of supporting electrolyte and pH:
The nature and acidity of the supporting buffer are some of the most important factors which strongly influence the stability of the analyte and its cathodic reduction and adsorption processes. Among the various investigated buffers (B-R, acetate, carbonate, phosphate and ammonia) the best voltammetric signal in terms of sensitivity (peak height) and resolution (peak shape) have been secured using B-R buffer. In addition, when the AdSV peak current was measured as a function of pH over 2-7 range, the stripping voltammetric signal increased steadily over the acidic region and the peak current reached its maximum value at pH 2.5 which was selected as optimal value for subsequent studies. It is noteworthy that when alkaline B-R supporting electrolyte was used, chlorotetracycline/Zn (II) complex was barely detectable and nearly no stripping voltammetric signal was observed. The variation of AdSV peak current with pH, obtained for 5x10^-7 mol l^-1 chlorotetracycline drug and 1x10^-6 mol l^-1 metal ion concentration accumulated for 40 sec is exhibited in Figure 4.

Effect of accumulation time and potential:
Preconcentration of the analyzed drug on the surface of HMDE is one of the essential conditions for highly sensitive determinations. Variation of the accumulation time over 0-150 sec period for 5x10^-7 mol l^-1 chlorotetracycline drug solution and 1x10^-6 mol l^-1 Zn^2+ solution at a preconcentration potential of 0.0 V, showed a gradual enhancement for the monitored peak current over the range 0-40 sec. The dependence of peak current on accumulation time is presented in Figure 5. The proportional relationship was nearly observed up to 40 sec and then it became virtually curved and leveled off owing to the saturation of the hanging mercury drop by the analyte. For further experiments an accumulation time of 40 sec was selected as optimal because it provided relatively high peak current with adequate practical time. The variation of accumulation time did not produce significant shifts in peak potential value.
In addition, as can be seen from Figure 6, when the influence of accumulation potential on the monitored electrochemical response was examined over the -0.8 to +0.4 V range at 40 sec pre-concentration time, the peak current increased steadily over the positive direction till it reached its maximum value at $E_{ac} = -0.4 \text{ V}$ where it decreased sharply after potential -0.4V. Thus, $E_{ac} = -0.4 \text{ V}$ will be adopted as optimum operational value for the following works as it ensured the highest AdSV signal.

**Effect of scan rate:**

The cathodic peak current of chlorotetracycline drug was found to be directly proportional to the scan rate, particularly at low scan rate values, a phenomenon characterized for adsorbed materials (21). When the AdSV peak current of $5 \times 10^{-7} \text{ mol l}^{-1}$ chlorotetracycline drug and $1 \times 10^{-6} \text{ mol l}^{-1}$ zinc ion in pH 2.5 B-R buffer was measured over the range 100-1000 mV/s, it was found that peak height was observed in the scan rate 600 mV/s. However, after this maximum value the peak current started to decrease slightly with faster scan rates. Accordingly, 600 mV/s scan rate value was adopted as optimum condition for further investigations.

**Effect of pulse amplitude and frequency:**

In addition, the impact of varying the excitation wave pulse amplitude on the voltammetric current intensity was also evaluated. The effect of this operating variable was studied over the range 10-100 mV (see figure 7) and it was concluded that in order to assure maximum peak current, 60 mV pulse amplitude was the ideal choice for this operational parameter. Moreover, varying the value of square wave frequency also plays an important role for the measured signal of SW-AdSV approach. When the AdSV peak current of $5 \times 10^{-7} \text{ mol l}^{-1}$ chlorotetracycline drug and $1 \times 10^{-6} \text{ mol l}^{-1}$ zinc ion in pH 2.5 B-R buffer was measured over the range 10-100 Hz (see Figure 8), it was found that peak height was quasi-linearly dependent on the frequency over the range 10-90 Hz. However, after this maximum value (90 Hz) the peak current started to decrease with increasing frequency. Accordingly, for further work 90 Hz SW frequency value was adopted.

**Effect of instrumental parameters:**

The monitored AdSV peak height could be further maximized by optimizing other experimental factors that can affect the adsorption process of the formed Drug/Zn complex. The influence of both the surface size of the mercury drop working electrode
and electrode convection rate was also evaluated. An increase in the surface of the working electrode (over 0.15-0.60 mm$^2$) yielded, as expected, a linear enhancement in the analytical signal and did not affect the value of the stripping voltammetric potential. In addition, An increase in the stirring rate (raising it from 0.0 to 3000 rpm) yielded, a linear enhancement in the analytical signal from 0.0 to 2800 rpm, after that it is decreased and did not affect the value of the stripping voltammetric potential. Thus, for optimal sensitivity, 0.60 mm$^2$ drop size and 2800 rpm stirring speed were chosen for subsequent practical works.

**Analytical Performance (Method Validation):**

Once the most ideal and suitable chemical conditions and instrumental parameters for the adsorptive determination were established, a calibration plot for the analyzed drug was recorded to estimate the analytical characteristics of the developed method.

**Calibration graph:**

Under the optimum conditions a very good linear correlation was obtained between the monitored voltammetric peak current and chlorotetracycline concentration in the range $1 \times 10^{-7}-1.4 \times 10^{-6}$ M, concentration of zinc ion $1 \times 10^{-6}$ M is constant in all measurements, (see Figure 9). Least-square treatment of the calibration graph yielded the following regression equation:

$$i_p (\text{nA}) = 120.25 + 1.42 \times 10^9 C \text{ (mol l}^{-1}) \quad \text{r =0.999, n = 7.}$$

where $i_p$ is the adsorptive stripping peak current, $C$ is the analysed drug concentration and $r$ is the correlation coefficient. The scale of the concentration range for this developed electroanalytical method SW-AdSV ($1 \times 10^{-7}-1.4 \times 10^{-6}$ M) is far wider than the $5\times 10^{-8}-5 \times 10^{-7}$ M linear concentration range reported previously (20).

**Detection limit:**

The lowest detectable concentration of this drug was $7.0 \times 10^{-9}$ mol l$^{-1}$, which was estimated based on the signal-to-noise ratio (S/N=3) and this obtained analytical sensitivity is very promising since it was achieved after employing very short accumulation time (40 s) comparing to that reported for the previous stripping voltammetric determination of chlorotetracycline using linear sweep technique which required 300 s accumulation time (20).

**Reproducibility:**

The high sensitivity of adsorptive voltammetry is accompanied by very good reproducibility. This analytical performance was evaluated from eight repeated measurements of electrochemical signal of $5 \times 10^{-7}$ mol l$^{-1}$ chlorotetracycline drug and $1 \times 10^{-6}$ mol l$^{-1}$ zinc ion solution. The precision of the electrochemical developed method in terms of the relative standard deviation (RSD%) was 3.4%.

**Accuracy:**

The accuracy of the proposed method was checked by calculating the recovery of known amount of chlorotetracycline ($3 \times 10^{-7}$ mol l$^{-1}$) and $6\times 10^{-6}$ mol l$^{-1}$ zinc solution added to B-R buffer solution and analysed via the optimized stripping voltammetric procedure. The value of the recovery obtained by the standard addition method was $99\%\pm 1.0$. The accuracy of the proposed SW-AdSV method was also verified further by the analysis of chlorotetracycline in real samples and comparing the obtained analytical results with that extracted from HPLC reference technique (see section: practical application) whereas no real practical applications were given for previous study (20).

**Stability:**

Under the optimum conditions, the stability of $5 \times 10^{-7}$ mol l$^{-1}$ chlorotetracycline and $1 \times 10^{-6}$ mol l$^{-1}$ Zn$^{2+}$ solution was evaluated by monitoring the changes in the height of AdSV peak over a period of
80 min. The electroanalytical signal was gradually constant with time. The acidic media (pH 2.5) of the B-R electrolyte solution probably initiated a slow degradation process for the drug.

**Interference Studies**

In order to evaluate the selectivity of the developed AdSV procedure, the influence of various interferences was examined. Considerable interference can be caused by co-existing surface-active compounds capable of competing with the analyte of interest for the adsorption site on the electrode surface, resulting in decreased peak height. The competitive co-adsorption interference was evaluated in the presence of various substance usually occur in the pharmaceutical tablets and formulations. For these investigations, the interfering species were added at different concentrations (one, 5-fold and 50-fold) higher than the concentration of chlorotetracycline (5 ×10⁻⁷ mol l⁻¹) and 1×10⁻⁶ mol l⁻¹ zinc ion. The addition of starch at these concentration levels caused the AdSV peak current to decrease by about 73.6%, 89.8% and 97.4%, respectively, of its original peak current. Apparently, this inhibition effect was caused by the working electrode surface blockage due to adsorption of interferences. Also the additions of 50-fold of cellulose and magnesium stearate in the test solution containing 5 ×10⁻⁷ mol l⁻¹ drug and 1×10⁻⁶ mol l⁻¹ metal ion, caused the AdSV peak current to decrease by about 24.2% and 65.5%, respectively. In contrast, the additions of lactose and sucrose at different concentrations (one, 5-fold and 50-fold) in the test solution containing 5 ×10⁻⁷ mol l⁻¹ drug and 1×10⁻⁶ mol l⁻¹ metal ion, caused no significant effects on the SW-AdSV response of the drug.

**Practical Applications**

The reliability of the proposed AdSV method for the determination of chlorotetracycline drug was investigated by assaying this drug in some real samples. Because of there is not chlorotetracycline drug in local market, using tetracycline drug instead of this drug. Following the developed electroanalytical procedure described above, tetracycline drug was analysed in pharmaceutical formulation. The tetracycline content of commercially available capsule (tetracycline – 250 mg tetracycline hydrochloride) was determination directly by the SW-AdSV method after the required dissolving and filtration steps. Four aliquots of the dissolved sample were diluted to the required concentration level and measured via the standard additions approach (conc. of zinc ion 5 ×10⁻⁶ M ). For these studies, results obtained gave a recovery mean 102.33% with standard deviation of ±0.58%. As can be seen from Table 1, these results achieved by the optimized AdSV procedure were in good agreement with those obtained by HPLC technique (the chromatographic analytical results and measurements were carried out in our lab) for the analysis of the same pharmaceutical capsule (tetracycline-250mg) manufactured by Julphar, U.A.E. Based on the statistical evaluation F-test approach (22) for these results, there is no significant difference between the results obtained by the developed AdSV procedure and that obtained by the reference method. When comparing the variances of the developed AdSV procedure and the chromatographic reference method (HPLC), the calculated F value is 2.97. Whereas the calculated F-test value (2.97) was less than the critical value (19) at the 95% confidence level. There is no statistical evidence that the variance of the proposed method differ significantly from the variance of the reference method. Furthermore, the agreement of the compared results obtained by the developed electroanalytical method and the HPLC reference method was also tested by the paired t-test statistical approach. The statistical means of both analytical methods were found to be not differ significantly, since the calculated t-test value (1.99) was less than the critical value (2.78) at the 95% confidence level (P = 0.05).

**Table 1. Analysis of tetracycline drug in its commercial capsule.**

<table>
<thead>
<tr>
<th></th>
<th>AdSV Method</th>
<th>Reference Method (HPLC)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Found (mg)</td>
<td>Recovery%</td>
</tr>
<tr>
<td>Labeled</td>
<td>255</td>
<td>102</td>
</tr>
<tr>
<td>Content</td>
<td>255</td>
<td>102</td>
</tr>
<tr>
<td>250 mg</td>
<td>257.5</td>
<td>103</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>102.33</td>
<td>±0.58</td>
</tr>
</tbody>
</table>

In addition, the applicability of the AdSV procedure for the analysis of chlorotetracycline drug in
biological samples was also evaluated by estimating its recovery from spiked human urine serum sample. A simple and fast pretreatment (clean-up) procedure, which is in fact a slight modification of the sample preparation method develop for the determination of some antagonist drugs (23) was used. By adding a small amount of 5% ZnSO₄·7H₂O solution, NaOH and methanol to the serum sample and centrifuging the mixture, most of the interfering substances (mainly proteins) were simply removed and eliminated by precipitation. As can be extracted from Table 2, this AdSV method (after appropriate dilution) allowed the determination of chlorotetracycline drug in serum sample with mean recoveries 103.6% ±0.55, respectively.

**Table 2.** Analytical result for chlorotetracycline drug recovery from serum sample.

<table>
<thead>
<tr>
<th></th>
<th>Spiked Serum % Drug Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Added</td>
<td>103</td>
</tr>
<tr>
<td>Chlorotetracycline</td>
<td>104</td>
</tr>
<tr>
<td>3.0 x 10⁻⁷ mol l¹</td>
<td>104</td>
</tr>
<tr>
<td>Mean</td>
<td>103.6</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>±0.55</td>
</tr>
</tbody>
</table>

**Acknowledgement**

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**References**

23. Stubauer G. and Obendorf D., Determination of trace levels of niguclidine in urine and blood by adsorptive stripping voltammetry at the hanging mercury drop electrode, Analyst 1996: 121, 351-56.